Combination of Formaldehyde with Casein

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Graphs are presented to show the effects of concentration of formaldehyde, pH, time, and temperature on the amount of recoverable formaldehyde remaining in combination with casein after exhaustive washing of the reaction product with distilled water. The results are compared with related data of other investigators and are discussed in terms of possible reactions of various structural units in the protein. The analytical procedures employed for distillation and titration of recoverable formaldehyde were extensively studied and improved. Experiments are also described that show appreciable conversion of formaldehyde to the nonrecoverable form in the presence of casein at 100° C. and above.

THE prevailing use of formaldehyde as a hardening agent in the manufacture of plastics and textile fiber from casein makes it important to know the extent to which formaldehyde is bound by this protein under various conditions. Immersion of casein or other protein in a solution of formaldehyde leads to combination of the latter with varying degrees of stability, which are not easy or possible to distinguish quantitatively. Nevertheless, the formaldehyde taken up by the protein can be classified as to (*a*) whether it is in sufficiently loose form to be dissipated by washing with cold water or by exposure to air, or (*b*) whether it is held firmly enough to resist prolonged washing. Measurements of the total amounts of formaldehyde taken up by proteins or allied materials while in equilibrium with the formaldehyde bath obviously include formaldehyde bound in both manners. Such studies have provided valuable information concerning amino acids as well as proteins. Carpenter and Lovelace (*3*) have reported data for casein which represent the difference between the original and final formaldehyde concentrations of the treating bath. On the other hand, the formaldehyde responsible for improved properties in plastics and fibers must be the fraction retained under ordinary conditions of use—that is, the more firmly bound. The formaldehyde content of collagen, washed or pressed free of the loosely held material, has been extensively studied (*15*) by Highberger and co-workers and by Theis and associates, who employed distillation with acid to liberate the aldehyde.

The work reported in this paper was undertaken to determine the effects of the four major factors—concentration, pH, time, and temperature—on the amounts of formaldehyde firmly bound by casein, and, if possible, to correlate the results with amino composition. Nitschmann and Hadorn (*17*) have reported data on the effects of time and concentration but no series of results showing the effects of pH or temperature.

MATERIALS AND METHODS

Since the primary aim of this work was to study the reaction involved in the hardening of protein plastics and fibers, the protein selected was a high quality acid casein obtained commercially and ground to pass a 60-mesh screen. Preliminary experiments showed that particle size had a negligible effect over the range studied (40 to 150 mesh).

Air-dried casein equivalent to 2.00 grams on the dry, ash-free basis was added to 100 ml. of the buffered aqueous solution of formaldehyde in a large test tube. The solutions were 0.1 *M* with respect to phosphate ion. They were prepared by diluting the required amounts of 37 to 39% formalin and 0.5 molar KH_2PO_4 and K_2HPO_4 solutions (or, where necessary, H_3PO_4 and KOH) to give the desired pH and formaldehyde concentration. Initial and final pH values were determined with a glass electrode. An original pH of solution of 4.8 remained the same during the reaction with casein, whereas a pH of 6.0 declined to 5.6 ± 0.2, and higher pH values dropped more markedly. Formaldehyde concentration was determined by the hypoiodite method of Romijn (*20*). The concentration change of formaldehyde in solution during the reaction was only a small fraction of the total.

Care was exercised to obtain even wetting of the casein particles and to avoid the formation of aggregates. The tubes were stoppered tightly and immersed in a constant temperature water bath equipped with a revolving carriage which turned them end over end continuously. Temperature control was accurate to ±0.5° C.

Each of the factors—concentration of formaldehyde, pH, time, and temperature—was studied over a wide range under standardized conditions. The standard conditions chosen were pH 6.0 (initial), 25° C., 24 hours, and initial concentration of formaldehyde of 4.5 grams per 100 ml. Two to four (usually four) samples were carried through the entire procedure under identical conditions for each point of the whole range studied.

Shortly before the end of the reaction period the solid was allowed to settle, and the supernatant liquid was set aside for pH and formaldehyde determinations. The hardened casein particles were immediately resuspended in distilled water. This point was taken as the end of the reaction time. Washing by decantation was repeated several times, after which the solid was transferred to a 200-ml. bottle equipped with a rubber stopper carrying two filter tubes arranged so as to permit continuous flow of water in either direction. Several such extractors were connected to a single water reservoir by means of a feeder bottle. A slow stream of distilled water was passed through the extractor bottles, with only occasional agitation, until a 200-ml. portion which had remained in contact with the casein for at least an hour gave a negative test for formaldehyde with a sensitive Schiff's reagent. Small amounts of the remaining formaldehyde could be progressively removed by further washing but only at an extremely slow rate. The Schiff's reagent was adjusted to give a positive test with 0.001 to 0.01% formaldehyde solution and was checked frequently against solutions of known strength. Except for the removal of very large amounts of excess formaldehyde, the passage of about 5 gallons of water per 2-gram sample during 2 to 3 days was usually adequate. A more rapid rate of flow did not greatly increase the rate of removal. The washed samples were filtered and dried, first for about 30 minutes at 50° C., then at room temperature overnight.

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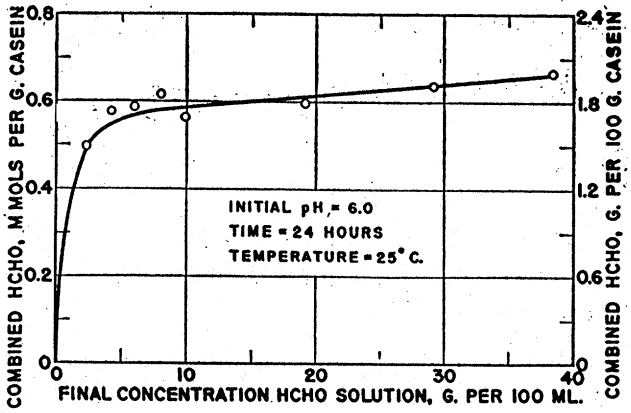

Figure 1. Effect of Concentration of Formaldehyde on Combination of Formaldehyde with Casein

ANALYSIS FOR COMBINED FORMALDEHYDE

Combined formaldehyde was determined by a modified method based on that developed by Nitschmann and Hadorn (*16*), who applied to hardened casein much the same procedure used by Highberger and Retzsch (*13*) for formaldehyde-treated collagen. Formaldehyde is distilled in the presence of acid into bisulfite, and the combined bisulfite equivalent to the formaldehyde is determined iodometrically. Nitschmann and Hadorn substituted 0.1 *M* phosphoric acid for 2 *N* sulfuric acid (*13*). Later with Lauener (*18*) they recommended a second distillation after the residue was diluted with water, and in the case of hot-hardened casein, even a third distillation with 1.8 *M* phosphoric acid. We used 0.1 phosphoric acid but discovered, as did the Swiss workers, that a second distillation was necessary for samples containing large amounts of formaldehyde. In fact, we found that both 2 *N* sulfuric acid and 0.1 *M* phosphoric gave satisfactory results when the samples were adequately distilled. The titrimetric features of both methods (*13*, *16*) referred to depend for their success upon principles elucidated in 1927 by Friedemann, Cotonio, and Shaffer (*10*), who made an exhaustive study of the iodometric procedure for titrating acetaldehyde-bisulfite solutions first devised by Clausen (*4*). We examined critically the results of these various investigators and, after the introduction of several modifications, finally developed a titration method which was satisfactory in our hands.

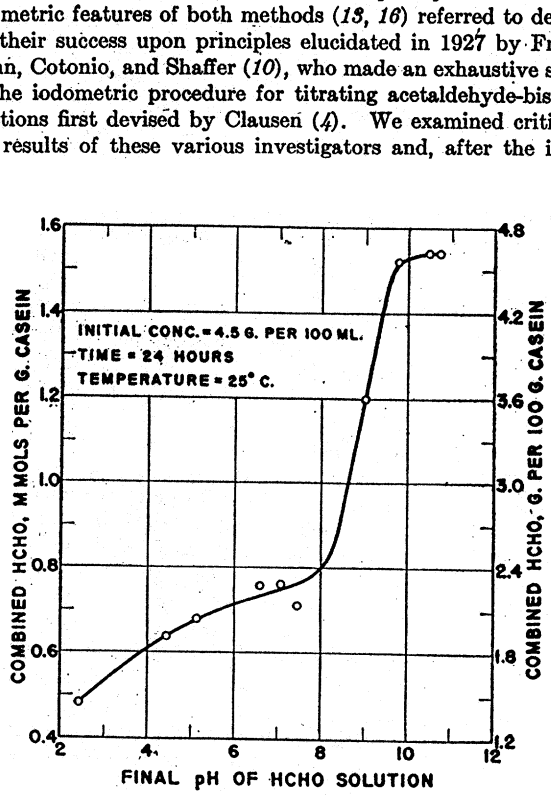

Figure 2. Effect of pH on Combination of Formaldehyde with Casein

The distillation apparatus was a 500-ml. Kjeldahl flask connected by a rubber stopper to a one-piece condenser tube having two right-angle bends and leading through a vertical water jacket to the bottom of the receiver, a 250-ml. volumetric flask. The latter contained 12 to 15 ml. of 1% aqueous sodium bisulfite solution (at least a twofold excess). A quantity of the hardened casein sample roughly equivalent to 0.2 to 0.4 gram of anhydrous material, and containing 1 to 12 mg. of combined formaldehyde and 30 to 60 mg. of nitrogen, was transferred to the Kjeldahl flask and covered with 200 ml. of 0.1 *M* H_3PO_4. The distillation rate was adjusted so that about an hour was required to reduce the volume of liquid in the Kjeldahl flask to about 10 ml.

At the end of the distillation the condenser was washed down and the distillate was diluted to 250 ml. with distilled water, mixed thoroughly, and allowed to stand for at least 15 minutes before titration. When more than one distillation was necessary to obtain complete recovery of combined formaldehyde, the residue in the Kjeldahl flask was diluted to about 200 ml. with distilled water, and a fresh receiver containing 12 to 15 ml. of 1% bisulfite solution was put into place. Distillation was carried out as before; the second distillate was diluted to the mark and titrated in exactly the same way as the first. In casein samples containing 2% or less of combined formaldehyde, the amount obtained in the second distillation was usually not more than 5% of the total, and no additional formaldehyde appeared in a third distillation. Occasional samples containing 3.5 to 4.0% of combined formaldehyde gave as much as 15 to 20% of the total in the second distillation and an additional 2 to 3% in a third.

After the removal of combined formaldehyde, generally by two distillations, the residue in each flask was submitted to a Kjeldahl procedure which was shown to yield all the nitrogen. Results were expressed in terms of nitrogen in the entire sample and were converted to the corresponding weights of casein by use of the factor 6.38. Calculation with this factor is on the basis of 15.65% nitrogen, which is the average of the best values for purified casein. The casein used actually contained only 15.0% nitrogen on a moisture-ash-free basis.

For titration of the distillates in the formaldehyde determination, aliquots of 50 ml. were measured by pipet and mixed with 5 ml. of 0.5% starch solution. Excess bisulfite was exhausted by running in 0.1 *N* iodine solution until near the end point, then titrating to a faint blue with 0.01 *N* iodine solution. The liberation and titration of combined bisulfite, after this preliminary removal of the excess, requires a special technique under standardized conditions for satisfactory results. The bisulfite must be titrated as rapidly as it is set free, but without an appreciable excess of iodine at any time. Since over a narrow range the speed of dissociation of the bisulfite complex increases with rising pH, the rate of titration can be controlled rather closely by adjustment of the pH in this range. This may be accomplished as follows: To the pale-blue solution resulting from the preliminary titration of excess bisulfite, add about 1 gram of solid sodium bicarbonate and mix. The blue color takes on a pink cast and fades. At once begin the dropwise addition of 0.01 *N* iodine solution from the buret; at the same time add from a cylinder 1 or 2 ml. of 5% sodium carbonate solution. The rate of liberation of bisulfite immediately increases, and the addition of iodine solution must be speeded up correspondingly. As the end point is approached, the rate diminishes; more sodium carbonate solution may then be required to obtain complete dissociation of the complex within a reasonable time, but no more than 10 ml. in all should be added during the titration.

The end point is not the typical blue but the faintest pink or lavender obtained by the addition of no more than one drop of 0.01 *N* iodine solution. This color is easily observed against a

white background and is stable for several hours at the pH (9.2) reached at the end of the titration described. If desired, an additional 10 ml. of 5% sodium carbonate solution may be added as a check to ensure complete dissociation of the formaldehyde-bisulfite complex, to bring the pH to about 9.5. At this more alkaline reaction the color is somewhat less stable, but no immediate fading occurs if the end point has been reached at the lower pH. Rapid fading does occur, however, at pH values of about 10 or above, because of side reactions which slowly consume iodine. It is therefore important that the amount of excess bisulfite reacting in the preliminary titration, during which acid is produced, and the amounts of bicarbonate and carbonate added be standardized within the limits specified. The procedure is applicable to larger amounts of formaldehyde, provided appropriate adjustments are made. Unless the precautions described are observed, highly variable results are obtained, no doubt as a consequence of extraneous oxidation of liberated bisulfite by dissolved air as well as various side reactions which consume iodine.

Results were calculated to weight of formaldehyde in the entire sample (1 ml. of 0.01 *N* iodine = 0.15 mg. formaldehyde). A blank of 0.10 ml. of 0.01 *N* iodine was subtracted from the titration value for the 50-ml. aliquot, since this amount of iodine solution was required in the absence of bisulfite to give a detectable color under the conditions of the titration. The determination of bisulfite-binding substance in unhardened casein by acid distillation gave essentially this same blank value.

Check experiments designed to test the recovery of a known amount of formaldehyde by the procedure just described showed that: (*a*) When casein is omitted, a single distillation gives complete recovery of amounts of formaldehyde (4 to 20 mg.) covering the range for which the method was designed; (*b*) when an amount of formaldehyde in this range together with 0.2 to 0.4 gram of casein is added to the distillation flask and distillation is carried out immediately, complete recoveries are again obtained; (*c*) when about 0.3 gram of casein and 20 mg. of formaldehyde (1 ml. of approximately 2% solution) are allowed to stand at room temperature in a closed Kjeldahl flask for several days before distillation, in order to allow combination, recovery of 97 to 99% of the total amount of formaldehyde present is obtained by exhaustive distillation. The maximum amount of formaldehyde lost under these latter conditions is equivalent to 0.2 gram per 100 grams of casein. This is doubtless not entirely a loss in recoverable combined formaldehyde, since there is probably some slow conversion to a nonrecoverable form. Experiments showing that more drastic conditions increase the size of this fraction are presented in a subsequent section.

The points plotted in the accompanying charts represent averages of two to four (generally four) replicate experiments. The largest deviation of the individual result from the corresponding average value was in general never greater than 0.03 millimole of formaldehyde per gram of casein.

DISCUSSION

The experimental data are presented in Figures 1 to 4. The only extensive data of a similar nature found in the literature are those of Nitschmann and Hadorn (*17*). They treated purified casein with formaldehyde at about 17° C. and pH 4.7 to 5. Air samples were exhaustively washed with water, generally for 12 days, and then analyzed for formaldehyde by their distillation method. Results were expressed as percentage of the formaldehyde-casein product on the dry weight basis. For 24-hour periods and bath concentrations of formaldehyde of 2, 10, and 38%, the respective amounts of formaldehyde bound were 1.14, 1.80, and 1.91%. These values fit the graph of Figure 1 remarkably well. Nitschmann and Hadorn also studied the effect of time, using 1-, 9-, and 28-day periods. When the bath concentration was 10%, the formaldehyde fixed by the casein

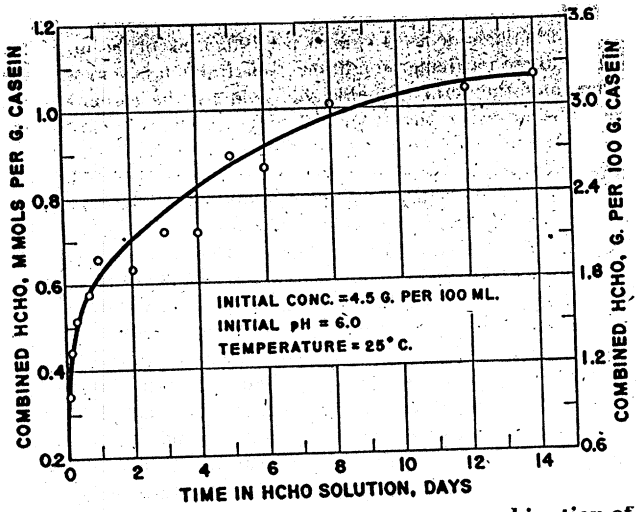

Figure 3. Effect of Time of Reaction on Combination of Formaldehyde with Casein

amounted to 1.80, 1.99, and 2.33%, respectively. The first of these values agrees well with the present data (Figure 3), but the second figure—1.99% for 9 days—is appreciably lower than the corresponding value in our graph. This difference may be due to the accumulating effect over 9 days of their somewhat different experimental conditions.

It is of some interest to compare our results with those obtained in studies in which measurements were made of the total formaldehyde held by casein in equilibrium with the formaldehyde solution. Carpenter and Lovelace (*3*) equilibrated 5-gram samples of acid casein with 25 cc. of formaldehyde of various concentrations at 25° C. and pH 7 (or slightly less). The amounts of formaldehyde held by the casein were determined by difference of the original and final concentrations of formaldehyde in the bath. It may be calculated from their data, which they found to fit Freundlich's adsportion equation, that casein immersed in a 4.5% solution of formaldehyde (44 grams per 1000 grams of solution) would take up 6.9 grams of formaldehyde per 100 grams of casein. Our time curve (Figure 3) shows that when acid casein is subjected to 4.5% of formaldehyde at 25° C. and pH 6 for sufficient time to give a nearly constant amount of formal-

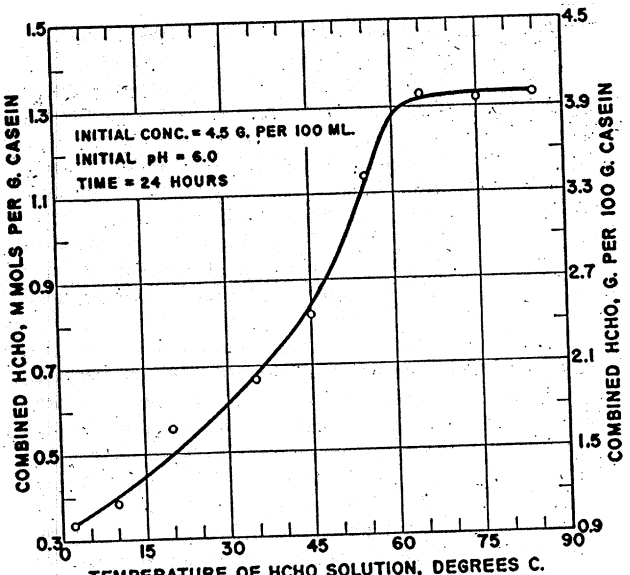

Figure 4. Effect of Temperature on Combination of Formaldehyde with Casein

dehyde binding after exhaustive washing, the bound but recoverable formaldehyde is 3.15 grams per 100 grams of casein. Thus, under these particular conditions, this fraction is somewhat less than half the total formaldehyde held by the casein when in equilibrium with the formaldehyde solution. A part of the formaldehyde represented by the difference between these two sets of values is probably nonrecoverable by distillation with acid. Nonrecoverable formaldehyde was not determined for the conditions employed in the experiments reported in Figures 1 to 4, but we assume that the amount formed at room temperature during 24 hours was relatively small. It may, nevertheless, be important in the hardening process.

The experiments on casein presented in Figures 1 to 4 may also be compared with similar ones performed by Highberger and Retzsch (*14*) on collagen. Variables studied by them were time, concentration, and pH, and graphs of their results have the same general shapes as our graphs for casein. Of particular interest is the fact that the graphs of bound formaldehyde plotted against pH show a distinct inflection at about pH 7 or 8 for both proteins. In general, under similar conditions, collagen binds less formaldehyde than does casein.

It would be desirable to predict closely the amount of formaldehyde bound by casein or other protein on the basis of the amino acid composition and the behavior of each type of structural unit under given conditions. Examination of the literature (*9*) shows, however, that analytical separation of the contributions of various reacting groups in a complex protein is elusive and that calculations of the kind suggested must for the present be limited to approximations. What appear to us to be the principal developments in this field are as follows:

The reaction of the amino group with formaldehyde, both in amino acids and proteins, has been demonstrated by several methods, which differ in their approach. Experiments on amino acids in equilibrium with formaldehyde have shown that the epsilon amino group (of lysine), as well as the unsubstituted alpha amino group, can bind one or two moles of formaldehyde, depending on the concentration of the latter. The first mole is more firmly bound than the second, and this fact is qualitatively consistent with the partial loss of formaldehyde from treated protein upon washing. Besides lysine, other amino acids—arginine, histidine, asparagine, glutamine, tryptophane, cysteine, and even threonine and serine—have side chain groups which might react with formaldehyde. Several of these amino acids, when treated with formaldehyde under conditions of varying severity, are known to undergo internal cyclization through the alpha amino group, but the results are of restricted application to proteins, which contain relatively few unsubstituted alpha amino groups. Evidence for the participation of the guanidine group in the binding of formaldehyde by the washed protein product has recently been strengthened by the finding of Fraenkel-Conrat and Olcott (*8*), that protamine in 4 days at pH 7.6 binds as much as two moles per arginine unit at 70° C. and about one mole at pH 3.2. Uptake at room temperature was less than half that at 70° C. In view of the report of Frieden, Dunn, and Coryell (*11*), that formaldehyde upon standing with arginine for 20 to 30 minutes prevents the typical carmine color of the Sakaguchi reaction, the present authors performed similar experiments with casein. If a solution of casein at pH 10 is treated with an excess of formaldehyde for 24 hours, the test yields a yellow color, nearly the same as that obtained in a formaldehyde control. If the color reagents are added immediately after the formaldehyde, the carmine color ensues, although it is not so intense as that given by the casein control. This apparent reaction of formaldehyde with the guanidine group of the casein, as gaged by prevention of a characteristic color test, proceeds nearly to completion at pH 8 and is marked at a pH as low as 6. Information in regard to the reaction of imidazole, indole, hydroxyl, and sulfur-containing groups to give products containing recoverable formaldehyde is less decisive. Evidence for the reaction of amide groups in proteins with formaldehyde has been presented by Wormell and Kaye (*21*) and Fraenkel-Conrat, Cooper, and Olcott (*7*). The latter authors found that polyglutamine in 4 days at 70° C. bound wash-resistant formaldehyde equivalent to half the amide groups at pH 6.7 and equivalent to three quarters of the amide groups at pH 3.5. There is no experimental proof that the peptide group reacts extensively with formaldehyde. If such reaction did occur on a large scale, all formaldehyde-treated proteins should show definitely larger quantities of stable formaldehyde than have been found. Moreover, such model substances as polyglycine, polyglutamic acid, and nylon (*7*) as well as benzoyl alanine (*2*) do not indicate any considerable activity of the peptide groups.

If it is tentatively assumed that each lysine, arginine, histidine, and amide unit of casein binds one molecule of formaldehyde, the amounts bound would be the sum of 0.54, 0.21, 0.19, and 0.94 millimole of formaldehyde per gram of casein, respectively, or a total of 1.88 (calculations based on recent amino acid figures checked by different methods). The highest values found for casein were a little over 1.3 in the temperature series and 1.5 in the pH series. Exposure of the casein to formaldehyde in these experiments was for 24 hours.

Increasing concentration of formaldehyde up to about 4 grams per 100 ml. causes a sharp increase of combined formaldehyde (Figure 1), and the effect of time (Figure 3) is greatest during the first 24 hours. In both cases there are inflection points, and the corresponding ordinate values for combined formaldehyde approximate 0.6 millimole per gram casein. Casein contains 0.6 millimole of free amino nitrogen. However, we do not regard this as necessarily more than a coincidence, inasmuch as the conditions used in these experiments were only one combination of many which might have been selected.

The theory that only the uncharged forms of basic groups in proteins react with formaldehyde has been widely adopted. It would thus appear that some minimum pH might be established at which, under standard conditions, combination could be expected to occur in an appreciable amount, and that this minimum pH would be lower than but related to the dissociation of the basic group in question. The literature affords hardly more than qualitative evidence of the application of this theory to wash-resistant recoverable formaldehyde in proteins. In fact, combination of amino groups at pH 3.5 (*7*) and even at pH 1 to 2 (*12*) and of guanidine groups at pH 3 (*8*) has been reported. Reference has been made to our experiments showing reduction of the chromogenic capacity of the guanidine group of casein when treated with formaldehyde at pH 6. During the reaction of any particular basic group in protein with formaldehyde, equilibrium between charged and uncharged forms is shifted toward the latter by the presence of other groups capable of accepting positive charges and by high concentrations of formaldehyde. These factors may account for appreciable binding of formaldehyde in pH regions considerably below the respective pK values of the basic groups involved. Furthermore, the pK values of the products in the case of free amino acids may be as much as 3 pH units lower than those of the amino acids themselves.

The amide group has been reported to react with formaldehyde

TABLE I. HEATING OF CASEIN IN FORMALDEHYDE AT 100° C. FOR 3 HOURS

Casein Present (Dry Basis), Mg.	Formaldehyde Present, Mg.	Formaldehyde Distilled, Mg.	Nonrecoverable Formaldehyde		
			Mg.	Millimole/g. casein	G./100 g. casein
183.5	4.05	1.75	2.30	0.42	1.25
183.5	4.05	1.63	2.42	0.44	1.32
183.4	8.14	4.53	3.61	0.66	1.97
182.6	8.14	4.62	3.52	0.64	1.93
199.4[a]	16.25	11.84	4.41	0.74	2.21
188.9[a]	31.40	25.70	5.70	1.00	3.02

[a] Duplicate sample lost.

at temperatures of 35° to 70° C. to a greater extent under acid conditions than at higher pH (*7*, *21*). On the other hand, Fraenkel-Conrat has recently presented data (*8A*) to show that the amide group at room temperature reacts more rapidly under alkaline than neutral or acidic conditions. Our pH series of data (Figure 2) show a marked inflection at about pH 8. It would appear probable, in light of the considerations discussed in the preceding paragraphs, that this curve represents chiefly a composite of the action of the basic and amide groups, with the former if not also the latter exerting the greater effect above pH 8.

NONRECOVERABLE FORMALDEHYDE

In our early work on casein plastics it was suspected that hot molding of formaldehyde-treated material might result in alteration of the formaldehyde in such a manner as to make it nonrecoverable by acid distillation. This possibility was of interest, considering that the converted formaldehyde, if bound to the protein, could conceivably contribute to a more stable type of hardening. A variety of experiments were therefore performed on casein to determine to what extent nonrecoverable formaldehyde may be produced.

The first trials were made to explore the effect of boiling in 0.1 *M* phosphoric acid (used in analytical procedure) on the recovery of total formaldehyde. Duplicate samples of 0.42 gram casein were let stand for 3 days with 2 ml. of 2% formaldehyde, then refluxed for 8 hours with 200 ml. of 0.1 *M* phosphoric acid, and finally exhaustively distilled. The recovery was only 90%. Control experiments performed in exactly the same fashion except that no casein was present yielded 100% of the added formaldehyde. Obviously the conditions of distillation, when prolonged, lead to an unmistakable decrease in the recoverable formaldehyde fraction.

The effect of heating formaldehyde in the presence of casein but omitting the effect of the 0.1 *M* phosphoric acid was studied in another experiment as follows. Approximately 200 mg. of casein were weighed into each of a series of flasks of about 3-ml. capacity. Then exactly 1 ml. of formaldehyde solution of about 0.4, 0.8, 1.6, or 3.2% was introduced, and the flask, partly immersed in solid carbon dioxide, was sealed at the top. Duplicate flasks were prepared for each formaldehyde solution, the exact titer of which was measured by titration of separate samples. The flasks were all heated for 3 hours in a boiling water bath and allowed to cool. The total formaldehyde in each flask was then determined by breaking the flask under 0.1 *M* phosphoric acid in a 500-ml. distilling flask and completing the analysis by the procedure already described. Two or more distillations were run to exhaust the recoverable formaldehyde from the sample. The results, presented in Table I, again demonstrate the production of definite amounts of nonrecoverable formaldehyde, which increase with the concentration of total formaldehyde present. It is estimated that the values for the nonrecoverable fraction are accurate ±10%. Casein alone gave no measurable amount of formaldehyde.

It was further shown that a well washed preparation of hardened casein containing 1.69% of recoverable formaldehyde and 20% of water yielded only 0.26% of formaldehyde after heating in a sealed tube for 1 hour at 120° C. and less than 0.1% after similar heating at 150° C. No loss occurred when formaldehyde alone was heated at 150° C. The nonrecoverable formaldehyde is probably, but not necessarily, bound in some manner to the casein.

The possibility of nonrecoverable formaldehyde in protein has been suggested by Baudouy (*1*), who cited the analogies of irreversibly bound formaldehyde in the cyclized products of histidine and tryptophane. The potential role of these amino acids has more recently been emphasized by Nitschmann and Lauener (*19*). They estimated that casein hardened in formaldehyde gas at 70° C. may contain as much as 5% of nonrecoverable formaldehyde. Discounting the conversion of the latter to either methanol or to formic acid, they came to the conclusion that, in their experiments, hydrolysis products of casein containing histidine and tryptophane were mainly but not entirely responsible. As pointed out in a previous section, the formation of ring compounds from these amino acids and formaldehyde is dependent upon the free alpha amino group, which is practically lacking in protein. However, other types of reaction between formaldehyde and the indole or imidazole nucleus are possible. Direct evidence relating the tryptophane unit in protein to formaldehyde binding has now been obtained by Fraenkel-Conrat, Brandon, and Olcott (*6A*), who found that gramicidin, which contains 40% tryptophane, binds 1 mole of nonrecoverable formaldehyde per each tryptophane residue. Their results speak for a methylol group in position 2 on the indole ring and are in agreement with the expectation that the nonrecoverable formaldehyde would be bound in C-to-C linkage. [More recent results of these authors (*6*) indicate that the methylol group may be attached at position 1 of the indole ring in gramicidin and in proteins. However, the possibility remains that the failure of most of the formaldehyde to be liberated by distillation may be due to migration of the methylol group to the 2 position as a consequence of the treatment with hot acid.] On the other hand, combination of protein and formaldehyde in such a way that the latter is recoverable upon distillation with acid should be accountable on the basis of linkage with one or two atoms of nitrogen, oxygen, or sulfur (*5*). Thus there would be no necessary connection between nonrecoverability and cross linking of formaldehyde in protein. Our results (Table I) indicate that the nonrecoverable formaldehyde on an equivalent basis may considerably exceed the tryptophane and histidine contents of casein.

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